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Naval Medical Research Institute
Commanding Officer
8901 Wisconsin Avenue
Bethesda, Maryland 20889-5607

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B Cells From M167 μκ Transgenic Mice Fail to Proliferate After Stimulation with Soluble Anti-Ig Antibodies

A Model for Antigen-Induced B Cell Anergy[1]

Donna G. Sieckmann,[2]* Kevin Holmes,† Peter Hornbeck,‡ Erin Martin,* Gretchen Guelde,§ Subbarao Bondada,¶ Dan L. Longo,‖ and James J. Kenny§

*Naval Medical Research Institute, Bethesda, MD 20889; †Resources Branch and Laboratory of Immunopathology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; ‡Department of Medicine, Division of Rheumatology, University of Maryland at Baltimore, Baltimore, MD 21201; §Program Resources Inc./Dyncorp., National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD 21702; ¶Department of Microbiology and Immunology and Center on Aging, University of Kentucky, Lexington, KY 40536; and ‖Biological Response Modifiers Program, National Cancer Institute, FCRDC, Frederick, MD 21702

The transgenic (TG) mouse strain 207-4, carries μ^a + κ transgenes ligated to the anti-phosphocholine (PC) V_H1 and Vκ24 V region genes from the MOPC-167 myeloma. Although B cells from mice carrying these transgenes respond both in vivo and in vitro to thymus-dependent Ags, they failed to proliferate in response to soluble goat anti-μ Ab or other soluble anti-Ig reagents. On the other hand, B cells from the Sp6 μκ anti-trinitrophenyl TG mouse line proliferated normally after stimulation with soluble anti-μ. However, the 207-4 anti-PC transgene positive (TG^+) splenic B cells proliferated when stimulated with anti-μ, anti-idiotype, anti-allotype, or PC-conjugated to Sepharose beads. TG^+ B cells were also induced to proliferate when stimulated with anti-Lyb-2; thus, their defect may be restricted to signaling through sIgM. The lack of response to soluble anti-μ could not be reversed by addition of IL-4, by removal of T cells, by addition of anti-FcR Ab, or by stimulation with $F(ab')_2$ anti-μ. Thus, the failure to proliferate was not caused by active T cell suppression or FcR-mediated inhibition. In mixed cultures of TG^+ and transgene negative (TG^-) spleen cells, the TG^- cells were able to proliferate normally to soluble anti-μ, indicating that suppressive factors were not involved in the unresponsiveness of the TG^+ anti-PC-specific B cells. These studies suggest that B cells in the 207-4 anti-PC TG mice exhibit a defect in activation through their sIgM receptors, and this unresponsiveness may reflect a form of Ag-induced tolerance. *Journal of Immunology,* 1994, 152: 4873.

Membrane surface IgM (sIgM)[3] is a receptor for mitogenesis in B lymphocytes, inasmuch as heterologous and anti-μ specific mAbs are able to induce proliferation in B cells (1–3). The role that sIgM and sIgD play in the activation and/or tolerance of B cells has been the subject of many studies which have attempted to elucidate the biochemical mechanisms involved in the cascade of events that lead either to activation and Ab secretion or to tolerance of the stimulated B cells. The recent development of transgenic (TG) mice bearing rearranged Ig genes (4–8), the products of which are expressed as Ag-specific receptors on virtually every B cell, provides new opportunities to address the mechanisms and biochemical pathways involved in Ag induced B cell activation and tolerance.

The 207-4 μκ anti-PC TG mouse line produced by Storb et al. (5) expresses the transgene-encoded M167-Id on more than 97% of its B cells (6, 9). These B cells respond normally both in vivo (6) and in vitro (10) to the thymus dependent Ag, PC-keyhole limpet hemocyanin,

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[2] Address correspondence and reprint requests to Dr. Donna G. Sieckmann, Infectious Diseases Department, Naval Medical Research Institute, 8901 Wisconsin Ave., Bethesda, MD 20889-5607.

[3] Abbreviations used in this paper: sIgM, surface IgM; sIgD, surface IgD; PC, phosphocholine; TG, transgenic; TG^+, transgene positive; TG^-, transgene negative; PE, phycoerythrin; PI, phosphatidylinositol; L, light.

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and thus represent a unique source of Ag-specific, Id^+ B cells in which to analyze the mechanisms of Ag-driven or anti-Id-driven differentiation. We have recently shown that the B cells in these mice are clonally deleted in an Ag-specific, receptor-mediated manner when the M167 μκ anti-PC transgenes are co-expressed in the presence of the *xid* gene (11). Furthermore, in M167 μ-only TG mice, M167-Id^+ B cells, which arise by association of the M167 μ transgene with an endogenous Vκ24 L chain, are expanded 100- to 500-fold over the number expected from random expression of L chain genes (9). This selective expansion of M167-Id^+ B cells also appears to be an Ag-driven, receptor-mediated process. Thus, B cells having the same Ag specificity appear to be either clonally deleted or clonally expanded depending on the X chromosome phenotype of the mouse in which they develop.

The B cells that develop in the 207-4 μκ TG mice differ from those of normal mice in that, 1) they express high levels of the transgene-encoded product on their surfaces; 2) they express no sIgD; and 3) endogenous encoded IgM is expressed on less than 20% of these cells (6). This cell surface phenotype is similar to that of immature B cells that have recently emerged from the bone marrow (12). B cells exhibiting this phenotype are more susceptible to tolerance induction than mature sIgM:sIgD-positive B cells (13). In the studies presented in this manuscript, we have analyzed the B cells from the 207-4 TG mice for their ability to respond to anti-Ig signals that induce proliferation in normal B cells. The results of these studies revealed a defect in the ability of B cells from 207-4 mice to proliferate in response to soluble anti-Ig-Abs even though they proliferate in response to the same Abs conjugated to Sepharose beads. Because this proliferative defect was not observed in the B cells from the μκ anti-TNP Sp6 TG mouse line or when the 207-4 B cells were stimulated through other mitogenic receptors, our results may indicate a selective tolerance mechanism in the PC-specific B cells, which results from a previous encounter with autologous or environmental PC during their early development.

Materials and Methods

Mice

TG mice carrying the MOPC-167 (M167) μ plus κ transgenes (line 207-4, designation Tg(Igh+Igk)Bri12), were obtained from Dr. U. Storb (Dept. Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL) through Dr. R. L. Brinster (School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA) and have been described previously (5). These mice are maintained in our breeding colony by backcrossing transgene positive (TG^+) male to C57BL/6 female mice. The Sp6 anti-TNP TG mice produced by Rusconi and Köhler (14) and characterized by Lamers et al. (15) were obtained from Dr. R. Hodes, National Institutes of Health, Bethesda, MD. The Sp6 μ^a + κ transgenes have been backcrossed onto a C57BL/6 background. These mice express the IgM^a transgene on all their B cells and co-express endogenous IgM^b and IgD on 10 to 20% of the B cells (15; J. J. Kenny, unpublished observations). The M167 μκ (207-4) mice do not express IgD (6). The transgene-encoded IgM^a is expressed at the same intensity on both Sp6 and 207-4 B cells (J. J. Kenny, unpublished observations). The progeny of both Sp6 and M167 TG mice are typed for the presence of the transgene by ELISA analysis of Abs bearing the IgM^a allotype (μ^a) (16). BALB/cByJ, C57BL/6J, and DBA/2J mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

Abs

Affinity purified goat anti-μ, goat anti-κ, and $F(ab')_2$ fragments of goat anti-μ Abs were prepared as described previously (2). Myeloma proteins MOPC104E (IgM,λ) and C.BPC112 (IgM,κ) were produced as ascites from myeloma cell lines provided by Dr. Michael Potter, National Cancer Institue, National Institutes of Health, through National Cancer Institute Contract CB05596–17 maintained with Hazelton Laboratories America, Rockville, MD. The mouse IgM mAb and myeloma proteins were purified from ascitic fluid by preparative centrifugation at 147,000 × *g* for 16 h, followed by chromatography on a Sepharose 6B column in a 0.01 M borate-buffered saline, pH 8.4.

AF6–78.25 (17), a mouse IgG1,κ mAb specific for the b-allotype of mouse IgM, and DS1 (16) an IgG1,κ mAb specific for the a-allotype of mouse IgM, were purified from ascites by precipitation with 50% saturated ammonium sulfate and passaged through a DEAE-Sephacel (Sigma Chemical Co., St. Louis, MO) column in 0.01 M Tris buffer pH 8.0. Anti-Lyb-2 was produced from ascites as previously described (18).

Abs were conjugated to cyanogen bromide-activated Sepharose 4B (Sigma) previously washed in 10^{-3} M HCl, at a ratio of 1 mg Ab protein to 1 ml packed volume of hydrated beads, in a 0.1 M $NaHCO_3$ buffer, pH 8 to 9. After overnight incubation on a rotating wheel at 4°C, the beads were washed in a 0.1 M $NaHCO_3$/NaCl buffer, blocked with 1 ml of 0.5 M ethanolamine pH 8 for 2 h at 4°C, washed with Dulbecco's PBS, and finally resuspended in Dulbecco's PBS with 0.02% NaN_3 at 1 part beads to 1 part buffer (1/2). The beads were sterilized by pasteurization for 1 h at 60°C. Before use in tissue culture, the beads were washed three times with MEM and resuspended in complete medium to the appropriate stimulatory dilution.

Cell culture and assay for [methyl-^{3}H]TdR ([^{3}H]TdR) incorporation

Spleen cells were cultured at 3×10^5 cells in flat bottom microtiter plates (Cluster 96, Costar, Cambridge, MA) in 0.2 ml of MEM containing 10% FCS, 16 mM HEPES buffer, 5×10^{-5} M 2-ME and appropriate concentrations of soluble anti-Ig or Sepharose 4B-coupled anti-Ig, Sepharose 4B-coupled anti-Id, or Sepharose-coupled anti-allotype, as previously described (2). IL-4 was the generous gift of Dr. William E. Paul, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD. DNA synthesis was measured after 48 h by a 16- to 18-h pulse of 1 μCi of 2 mCi/mM [^{3}H]TdR (Dupont NEN, Wilmington, DE). Cell cultures were harvested onto glass fiber filters and counted in a β-scintillation counter. Results are expressed as the geometric mean of triplicate cultures ± the SEM based upon a log normal distribution and calculated as previously described (2).

In experiments using purified B cells, T cells were removed from spleen cell preparations using the MACS Separator (Becton Dickinson, Palo Alto, CA). Spleen cells were incubated with biotinylated anti-Thy-1.2 (30H12), anti-CD4 (GK1.5), and anti-CD8 (53–6.72) Abs, followed by colloidal particles coated with streptavidin, and were passed over a C column according to manufacturer's instructions.

Flow cytometric analysis

Spleens were gently teased into single cell suspensions in HBSS without phenol red (GIBCO BRL, Grand Island, NY). Erythrocytes were removed by resuspending cells in 5 ml of ammonium chloride solution (M. A. Bioproducts, Columbia, MD) for 1 min and then washing three times in HBSS containing 5% FCS (HyClone Laboratories, Logan, UT) and 0.1% NaN_3 (HBSS + FCS). One hundred-microliter aliquots (10^6 cells) were prepared in 5-ml test tubes and 10 μl of FITC- or biotin-conjugated Ab (100 μg/ml) in HBSS + FCS was added. The cells were incubated for 30 min on ice and then washed twice in HBSS + FCS. Cells stained with a biotin-conjugated Ab were further reacted with phycoerythrin (PE)-conjugated streptavidin (Fisher Biotechnology, Silver Spring, MD) for 15 min at 4°C and washed with HBSS + FCS. The cells were then analyzed by flow microfluorometry as previously described (9).

Table I. *In vivo stimulation of 207-4 μκ TG spleen cells with anti-Ig Abs*[a]

Mitogen	TG+	TG−	BALB/cByJ	C57BL/6J
	(cpm per culture × 10^{-3} (± SEM)[b])			
Medium	5.2 (±0.7)	2.6 (±0.4)	6.2 (±0.6)	5.1 (±0.1)
Goat anti-μ	6.9 (±0.6)	59.7 (±3.2)	93.0 (±4.2)	114.0 (±5.1)
Goat anti-μ-Sepharose	79.2 (±1.2)	96.0 (±1.9)	162.0 (±6.3)	127.0 (±2.3)
Anti-μ^a-Sepharose	51.4 (±2.0)	2.6 (±0.3)	71.5 (±3.6)	5.0 (±0.2)
Anti-μ^b-Sepharose	5.4 (±0.2)	35.3 (±3.9)	5.3 (±0.2)	37.3 (±3.3)
Goat anti-κ-Sepharose	87.4 (±4.1)	102.0 (±0.4)	177.0 (±0.7)	118.0 (±5.0)
LPS	52.9 (±1.8)	54.5 (±0.5)	62.7 (±1.4)	106.0 (±1.5)

[a] Spleen cells from individual TG+ and TG− 207-4 mice were cultured in the presence of soluble goat anti-μ (100 μg/ml), goat anti-μ-Sepharose (1:150), goat anti-κ-Sepharose (1:100), anti-μ^a-Sepharose (DS1-Sepharose, 1:100), anti-μ^b-Sepharose (AF6-78.25-Sepharose, 1:100) as described in *Materials and Methods*.
[b] Data represent the geometric mean of triplicate cultures rounded off to two or three significant figures.

Table II. *Stimulation of anti-Thy-1.2 plus activated C-treated TG+ and TG− B cells from young and old mice*[a]

Stimulating Agent	TG+		TG−	
	Young	Old	Young	Old
	(cpm per culture × 10^{-3} (± SEM)[b])			
Media	0.4 (±0)	0.5 (±0)	1.5 (±0)	2.4 (±0)
Goat anti-μ	1.6 (±0.1)	2.0 (±0)	56.3 (±0.9)	93.5 (±10.1)
Goat anti-μ-Sepharose	27.3 (±1.4)	14.3 (±0.4)	93.9 (±8.3)	77.4 (±4.0)
Anti-V_H1-Id-Sepharose	267.0 (±32.7)	382.0 (±14.6)	1.9 (±0.1)	3.0 (±0.1)
Anti-M167-Id-Sepharose	155.0 (±5.2)	209.0 (±14.5)	1.8 (±0.1)	2.3 (±0.1)
PC-Sepharose	203.0 (±13.0)	487.0 (±67.6)	1.4 (±0.1)	2.1 (±0.1)
Rat-IgG-Sepharose	0.5 (±0.1)	1.0 (±0)	1.7 (±0.1)	2.3 (±0.2)
LPS	32.5 (±2.4)	25.1 (±0.9)	56.1 (±1.9)	51.5 (±3.0)
Con A[c]	2.5 (±0.1)	0.7 (±0)	5.6 (±0.5)	1.7 (±0.2)

[a] Spleen cells (10^7/ml) from old (16-mo) and young (4-mo) mice were treated with anti-Thy-1.2 plus C. After treatment, all groups of cells were greater than 80% B cells as determined by flow cytometry. Cells were then cultured and assayed as described in Table I.
[b] Data represent the geometric mean of triplicate cultures rounded off to two or three significant figures. This experiment was performed three times with similar results.
[c] Before anti-Thy-1.2 and C treatment, all groups showed in excess of 175,000 cpm after stimulation with Con A.

Results

Unresponsiveness of 207-4 TG spleen cells to stimulation by anti-Ig

Spleen cells from one TG+ and one TG− mouse were cultured with optimal stimulatory concentrations of soluble anti-μ, or Sepharose-conjugated anti-μ, anti-μ^a, anti-μ^b, or anti-κ Abs, and after 48-h incubation, cultures were analyzed for proliferation by [^{3}H]TdR uptake. As shown in Table I, spleen cells from the TG+ mouse were unresponsive to soluble goat anti-μ whereas the TG− cultures were stimulated 22-fold over the medium control. TG+ B cells also failed to respond to treatment with soluble anti-V_H1-Id and anti-M167-Id (28–5–15) at doses up to 200 μg/ml (data not shown). In contrast, the TG+ spleen cells gave a 15-fold higher response than the medium control after stimulation with the same preparation of goat anti-μ conjugated onto Sepharose beads. TG+ cultures also responded to goat anti-κ-conjugated Sepharose (Table I), anti-V_H1-Id, and anti-M167-Id conjugated to Sepharose (Table II), and LPS (Table I). However, the responses to these mitogens were lower than those given by the TG− spleen cultures, and the lower responses were probably caused by lower concentrations of B cells in the TG+ cultures, as discussed below.

The data in Table I also demonstrate that the TG+ and BALB/c spleen cells, but not the TG− or C57BL/6J cells, responded to anti-μ^a-allotype mAb conjugated to Sepharose. This was expected because the M167 H chain transgene encodes the μ^a allotype and all B cells in these mice express both the M167-Id and the μ^a-allotype markers on their surface (6, 9). As expected, the anti-μ^b-allotype mAb conjugated to Sepharose was mitogenic for the TG− and μ^b-allotype control C57BL/6 spleen cell cultures, but not for the TG+ or BALB/c cells. Less than 20% of the B cells in the TG+ mice co-express endogenous μ^b H chains on their surface (6). This may represent too few cells for effective stimulation in our culture system, or alternatively, the low density of μ^b chains on the surface may not permit effective signaling. Other spleen cell preparations from 207-4 TG+ mice have exhibited low levels of proliferation after stimulation with anti-μ^b-conjugated-Sepharose (data not shown). Overall, these results suggest that the transgene-encoded sIgM receptor on 207-4 B cells is capable of transducing mitogenic signals when stimulated by Sepharose conjugated anti-Ig, but not soluble anti-Ig.

The inability of the TG+ spleen cells to proliferate to soluble anti-μ was reconfirmed in nine additional experiments in which a total of 28 TG+ mice were individually

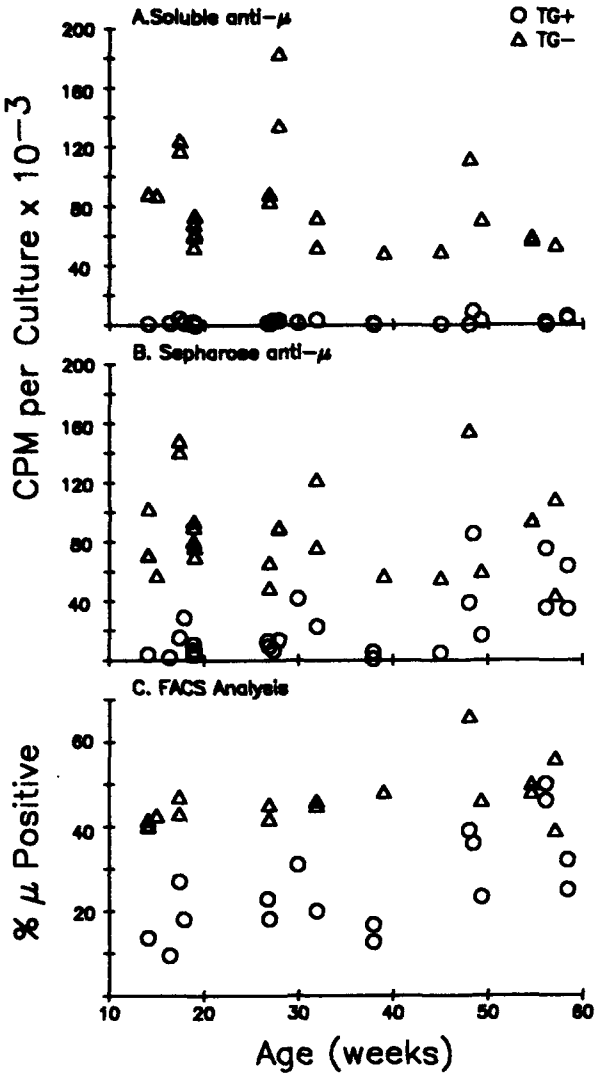

FIGURE 1. Response of individual TG^+ and TG^- mice to soluble or Sepharose-conjugated goat anti-μ and the total IgM-bearing B cells in spleens of TG^+ and TG^- mice. Data were compiled from nine experiments in which 26 TG^+ and 25 TG^- spleens were cultured individually at 3×10^5 cells/well with *A*, goat anti-μ (100 μg/ml) or *B*, goat anti-μ Sepharose (1:100). The geometric mean of triplicate cultures is plotted against the age of the spleen donor. In *C*, spleen cells of TG^+ and TG^- mice were individually stained with a FITC-conjugated goat anti-μ and analyzed by flow cytometry.

analyzed for their response to soluble and bead-conjugated anti-μ. The results in Figure 1*A* demonstrate that spleen cells from TG^+ mice 14 to 60 wk of age were unresponsive to soluble anti-μ. The data in Figure 1*B* show that these same TG^+ spleen cells responded at all ages, when stimulated by Sepharose-conjugated anti-μ, although the responses were found to increase with the age of the donor. The increased responsiveness with age is probably caused by the corresponding increase in total number of B cells present in older mice (Fig. 1*C*), because B cells from young mice proliferate as well as those from old mice, when T cells are depleted with anti-Thy-1.2 plus C′ treatment and the data are normalized with respect to the number of B cells placed in culture (Table II). The data in Figure 1*C* also demonstrate that the percentage of B cells in TG^+ spleens is generally less than that of the TG^- littermates.

To show that the lack of responsiveness of TG^+ spleen cells was not caused by a difference in optimum dose of soluble anti-μ required for stimulation of these cells and/or a difference in the kinetics of their response, spleen cells from TG^+ and TG^- mice were cultured in various concentrations of soluble anti-μ and the cells pulsed with [^{3}H]TdR at different times after stimulation. The TG^+ cells did not respond at any dose, whereas the TG^- cultures responded at all doses of soluble anti-μ tested; the TG^- cells continued to proliferate for 96 h, whereas the TG^+ cells did not proliferate during this same 4-day time period (data not shown).

Inability to show suppression by T cells, factors, or FcR mechanisms

The unresponsiveness of TG^+ B cells to soluble anti-Ig could possibly be caused by 1) FcR mediated inhibition; 2) T cell suppression; 3) suppressive factors; 4) sIgM receptor modulation; or 5) receptor-mediated cell death. It has been demonstrated that anti-μ-induced activation can be inhibited by the Fc portion of the Ab molecule acting through the B cell FcR (19). To investigate FcR-mediated inhibition as a possible cause of the unresponsiveness in TG^+ B cells, the spleen cells from TG^+ and TG^- mice were: 1) cultured with soluble goat anti-μ in the presence of various concentrations of purified 2.4G2 anti-FcR mAb (20), and 2) stimulated with goat $F(ab')_2$ anti-μ. The data in Table III demonstrate that anti-FcR Ab had no effect on the response of the TG^+ spleen cells or the control C57BL/6J spleen cells to soluble anti-μ, whereas the response of the TG^- spleen cells was enhanced an average of 1.7-fold in the presence of anti-FcR. Stimulation of TG^+ and TG^- spleen cells by $F(ab')_2$ anti-μ resulted in an increase of only 6300 cpm (at the highest concentration) in the proliferation of the TG^+ spleen cells, whereas the proliferation of TG^- spleen cells was enhanced by 29,000 cpm over the response obtained with an equal molar concentration of intact anti-μ Ab (Table IV). The low response by TG^+ spleen cells at the highest concentration of $F(ab')_2$ anti-μ was still three times lower than the response of the same cells to anti-μ-conjugated Sepharose and 10 times lower than the response to anti-V_H1-Id-conjugated beads (data not shown). The low response at the highest concentration of $F(ab')_2$ anti-μ may be caused by the stimulation of endogenous B cells in the cultures. Nevertheless, from these FcR blocking and $F(ab')_2$ stimulation experiments, it is evident that FcR-mediated inhibition is not the primary reason for the lack of anti-μ-induced responses in TG^+ B cells.

Table III. *Anti-μ stimulation of TG⁺ and TG⁻ spleen cells in the presence of anti-FcR Ab*[a]

Mitogens	Concentration of Anti-FcR (μg/ml)	C57BL/6[b]	TG⁺	TG⁻
		(cpm per culture × 10^{-3} (± SEM))		
Medium	Nil	4.5 (±0.1)	0.4 (±0.2)	1.4 (±0.1)
	125	5.5 (±0.5)	0.2 (±0)	1.5 (±0.3)
	250	7.8 (±0.2)	0.3 (±0.1)	3.7 (±0.5)
Goat anti-μ	Nil	86.2 (±1.1)	0.9 (±0.1)	20.1 (±2.9)
	125	97.6 (±3.1)	0.9 (±0)	63.9 (±1.7)
	250	86.5 (±1.9)	0.8 (±0.1)	67.4 (±5.7)
Goat anti-μ-Sepharose	Nil	129.0 (±9.7)	15.6 (±2.5)	63.6 (±3.2)
LPS	Nil	76.4 (±3.9)	24.3 (±2.4)	61.3 (±2.4)

[a] Spleen cells (3×10^5) of individual TG⁺ and TG⁻ mice were cultured in triplicate wells in the presence of soluble goat anti-μ (100 μg/ml) and 2.4G2 anti-FcR mAb, or Sepharose-conjugated goat anti-μ (1:200) or LPS (50 μg/ml) for 48 h before pulsing with [³H]TdR for 16 h. Results are represented as a geometric mean of the cpm/culture rounded off to two or three significant figures. Part of this data was previously published in different form in a review by J. J. Kenny et al. (21).

[b] This experiment was performed two times with similar results. In the other experiment, treatment of C57BL/6 spleen cells with 125 μg/ml 2.4G2 Ab in the presence of goat anti-μ, increased the proliferative response from 43,700 cpm to 86,800 cpm (experiment 1).

Table IV. *$F(ab')_2$ anti-μ stimulation of TG⁺ and TG⁻ spleen cells*[a]

Stimulating Agent	Concentration (μg/ml)	TG⁺	TG⁻
Media		3.6 (±0.2)	5.4 (±0.4)
LPS		43.8 (±3.4)	88.4 (±4.9)
Goat anti-μ-Sepharose		39.5 (±1.0)	77.7 (±3.5)
Goat anti-μ	100	6.1 (±0.4)	69.2 (±3.8)
	50	5.8 (±0.7)	52.9 (±2.9)
	10	2.8 (±0.4)	17.3 (±0.4)
	5	3.8 (±1.1)	5.4 (±1.4)
	1	2.8 (±0.4)	2.0 (±0.1)
$F(ab')_2$ anti-μ	61	12.4 (±0.7)	96.1 (±3.2)
	31	8.0 (±0.2)	69.7 (±9.8)
	6	5.5 (±0.2)	23.3 (±1.2)
	3	3.1 (±0.2)	7.2 (±0.3)
	0.6	3.0 (±0.3)	2.5 (±0.2)

[a] Spleen cells were cultured and data analyzed as described in Table I. Results are represented as a geometric mean of the cpm per culture × 10^{-3} rounded off to two or three significant figures ± SEM. This experiment was performed two times with similar results. Part of this data was previously published in a different form in a review by J. J. Kenny et al. (21).

As shown in Table II, the removal of T cells by treatment with anti-Thy 1.2 + C also had no effect on the inability of the TG⁺ B cells to respond to anti-μ. Because the anti-Thy + C treatment essentially eliminated the Con A responsive T cells, the presence of anti-μ-specific or nonspecific suppressor T cells in the TG⁺ spleen is unlikely. It was also possible that suppressor factors produced by the TG⁺ spleen cells were responsible for the failure of TG⁺ spleen cells to proliferate after anti-μ treatment. This was also ruled out by the data shown in Table V. When TG⁺ and TG⁻ spleen cells were cocultured in the presence of anti-μ, there was no suppression of the TG⁻ spleen cell response to soluble anti-μ; in fact, the addition of TG⁺ spleen cells to the TG⁻ cells resulted in a higher response than that obtained with irradiated filler cells.

Anti-Lyb-2-induced proliferation of B cells from TG⁺ mice

To elucidate whether the unresponsiveness of TG⁺ B cells to soluble anti-μ was limited to stimulation through the sIgM receptor, spleen cells from (207-4 × DBA/2)F1 TG⁺ and TG⁻ mice were cultured with soluble anti-Lyb-2 mAb (18). In two separate experiments, the mAb at 10 μg/ml stimulated both TG⁺ and TG⁻ spleen cells to proliferate, and when the response per B cell number is considered, anti-Lyb-2 stimulated the TG⁺ somewhat better than the TG⁻ cells. In one representative experiment, TG⁺ spleen cell cultures (39% B cells) responded with 11,300 cpm/culture (medium control—1,670 cpm/culture), and TG⁻ spleen cells cultures (60% B cells) responded with 10,400 cpm/culture (medium control—1,510 cpm/culture). Because this allospecific mAb stimulates B cells through a membrane receptor separate from the IgM receptor, these data demonstrate that the defect in the TG⁺ B cells is not a generalized hyporesponsiveness to stimulatory signals, but rather is limited to the membrane IgM receptor.

Addition of IL-4 does not rescue the anti-μ response of anti-PC B cells

IL-4 has been shown to function as a viability factor for B cells (22) and to synergize with suboptimum doses of anti-μ in the induction of B cell proliferation (23). The data in Figure 2 show that the addition of IL-4 to anti-μ-stimulated B cells has no effect on the TG⁺ B cells, whereas, the TG⁻ B cells exhibit an eightfold higher response to anti-μ plus IL-4 than to anti-μ alone.

Anti-μ-induced killing of B cells from 207-4 transgenic mice

From the above data, the unresponsiveness of TG⁺ B cells to soluble anti-μ appears to be caused by some inherent

Table V. *Spleen cells from M167 μκ TG mice do not suppress anti-μ-induced proliferation of normal spleen cells from TG⁻ littermates*

Cell Source[a] (×10⁻⁵)				Stimulating Agent			
Normal		Irradiated[b]					
TG⁺	TG⁻	TG⁺	TG⁻	Media	Anti-μ	Anti-μ-Sepharose	LPS
3	—[c]	—	—	2.1 (±0.1)	3.5 (±0.1)	56.7 (±0.4)	73.1 (±2.4)
—	3	—	—	2.9 (±0.1)	56.7 (±1.5)	135.0 (±3.6)	92.7 (±1.1)
1.5	1.5	—	—	3.6 (±0.2)	31.3 (±0.8)	101.0 (±7.1)	78.6 (±1.4)
—	1.5	1.5	—	1.4 (±0.1)	23.1 (±0.5)	82.3 (±5.8)	55.3 (±1.5)
—	1.5	—	1.5	1.2 (±0.1)	20.1 (±0.8)	75.0 (±3.3)	57.2 (±0.9)
—	—	3	—	0.1 (±0)	0.1 (±0)	0.1 (±0)	0.1 (±0)
—	—	—	3	0.1 (±0)	0.1 (±0)	0.1 (±0)	0.2 (±0)
1.5	—	—	1.5	0.8 (±0.1)	1.1 (±0)	32.9 (±0.8)	42.6 (±0.5)

[a] Spleen cells from individual TG⁺ or TG⁻ mice were cultured individually or in mixed cultures as described in Table I. Results are represented as a geometric mean of the cpm per culture ± SEM × 10⁻³. All data were rounded to two or three significant figures. This experiment was performed three times with similar results.
[b] Irradiated spleen cells received 3000 rad.
[c] —, no additions.

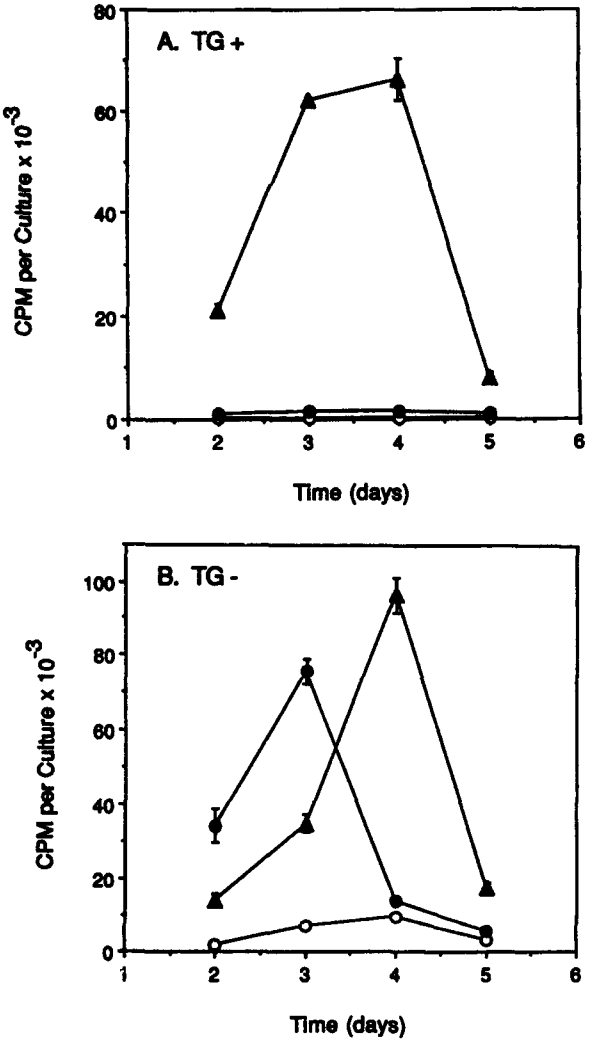

FIGURE 2. The unresponsiveness of B cells from M167 μκ TG mice cannot be reversed by IL-4. Purified splenic B cells from TG⁺ (*A*) and TG⁻ (*B*) mice were cultured with goat anti-IgM at 50 μg/ml (○), goat anti-IgM at 50 μg/ml and IL-4 at 1000 U/ml (●), or with LPS at 50 μg/ml (▲). Cells were pulsed with [³H]TdR for 4 h before harvesting.

property of the B cells rather than a result of external mediators. It has been shown that anti-μ treatment of neonatal B cells, which are predominantly sIgM only (24, 25), results in down modulation and failure to re-express sIgM receptors (13), whereas, mature sIgM⁺sIgD⁺ B cells will re-express these receptors within 18 h of anti-Ig stripping (13). It was therefore possible that soluble anti-μ was causing a similar receptor down-modulation on the IgM-only TG⁺ B cells; thus, no response would be seen because multiple rounds of anti-μ signaling are required to get effective induction of proliferation (1). Alternatively, it was possible that the anti-μ stimulation of TG⁺ B cells resulted in death of the cells rather than proliferation. To test both of these possibilities, TG⁺ and TG⁻ spleen cells were incubated with soluble goat anti-μ Ab, control goat IgG, or medium alone for 1 h at 37°C to allow binding and capping of the sIgM on the B cells. The cell suspensions were then washed, and a portion of the cells was stained with biotin-conjugated anti-B220 Ab plus either FITC-conjugated goat anti-μ, anti-μ[a], or rabbit anti-goat-IgG. Only low levels of goat Ab remained after the stripping and wash procedure (data not shown). The remainder of the cells were incubated overnight in RPMI 1640 + 10% FCS to allow regeneration of the membrane IgM and were then stained for B220, IgM and IgM[a]-allotype. The results of three experiments are shown in Table VI. The TG⁺ and TG⁻ spleen cell populations averaged 18 and 30.0% B cells, respectively. After 1 h incubation with soluble anti-μ, staining of sIgM was reduced to 1.3% and 3.5% in the TG⁺ and TG⁻ cultures, respectively; thus, sIgM was efficiently capped and removed from both cell types.

Staining of the TG⁺ cultures for IgM[a]-allotype gave essentially similar results. In both the TG⁺ and TG⁻ populations, the loss of B220⁺IgM⁺ cells was balanced by an increase in the number of B220⁺IgM⁻ cells. Twenty-four hours after treatment with goat anti-μ, all (44%) of the TG⁻ splenic B cells had re-expressed sIgM. In marked contrast, only 7% of the TG⁺ B cells had re-expressed

Table VI. *Goat anti-μ treatment of spleen cells from TG⁺ mice induces B cell death*[a]

		TG⁺			TG⁻	
	Treatment[b]	μ^- B220⁺	μ^+ B220⁺	μ^{a+} B220⁺	μ^- B220⁺	μ^+ B220⁺
		(percentage of total cells[c])				
Day 1	Untreated	0.9 ± 0.3	18.3 ± 2.7	16.7 ± 2.5	2.4 ± 1.1	30.7 ± 3.8
	Goat IgG	1.0 ± 0.2	18.0 ± 4.4	15.2 ± 0.1	1.4 ± 0.6	31.4 ± 3.0
	Goat anti-μ	16.2 ± 4.4	1.3 ± 1.0	2.4 ± 1.8	30.0 ± 2.5	3.5 ± 1.9
Day 2	Untreated	1.1 ± 0.4	20.2 ± 3.6	16.4 ± 5.2	1.5 ± 0.2	44.7 ± 4.5
	Goat IgG	1.1 ± 0.4	17.7 ± 0.6	13.1 ± 3.1	1.8 ± 0.1	47.1 ± 5.7
	Goat anti-μ	2.3 ± 0.9	7.2 ± 0.8	4.6 ± 1.4	3.3 ± 0.3	44.2 ± 6.0

[a] The results of three repetitive experiments are represented as the arithmetic mean ± SEM for each experimental value.
[b] Spleen cells (1 ± 10^7/ml) from TG⁺ and TG⁻ 207-4 mice were cultured in the presence of soluble goat anti-μ (100 μg/ml) for 1 h at 37°C, washed three times and cultured overnight at 37°C. In three experiments, cell recoveries from TG⁻ and TG⁺ cultures ranged from 24 to 42% and 27 to 33%, respectively. No significant difference in cell recovery was seen between anti-μ treated and untreated cultures.
[c] Spleen cells (1×10^6) were stained before and after anti-μ treatment with FITC-conjugated anti-μ, anti-μ^a, and biotin-conjugated anti-B220 plus PE-streptavidin and analyzed as described in *Materials and Methods*.

their surface IgM and very few B220⁺IgM⁻ cells remained in the culture. Inasmuch as 20% TG⁺ B cells remained in the medium control, these results indicated that approximately two-thirds of the TG⁺ B cells were induced to die within the first 24 h after anti-μ stimulation. The B cells that remained did not appear to down-modulate their receptors. When the same experiment was conducted with purified B cells, the TG⁺ cultures went from 80% B cells at time 0 to 49% 24 h later, whereas, the TG⁻ cultures went from 83% to 74% B cells in the same time period. These findings suggest that the failure of TG⁺ B cells to proliferate after anti-μ stimulation could be caused by preferential killing of these sIgM-only B cells; however, these receptor-regrowth experiments do not duplicate the culture conditions used in the proliferation studies described above in which the anti-μ Abs remained in the culture throughout the 48-h culture period. Therefore, bulk cultures of purified TG⁺ and TG⁻ B cells were incubated continuously with anti-μ and samples were removed and analyzed for viable cell recovery at 4, 24, and 48 h. The data from two experiments in Figure 3 show that under conditions of continuous anti-μ stimulation there was no difference in the viable cell recovery of TG⁺ and TG⁻ B cells from these cultures; however, the slope of the regression lines was always slighly greater in the presence of anti-μ. The results of these experiments suggest that induction of apoptosis by anti-μ cannot account for the lack of a proliferative response in the 207-4 TG⁺ B cells.

Proliferation of μκ anti-TNP TG splenic B cells after stimulation with soluble anti-μ

The restricted anti-μ stimulation defect observed in the B cells from 207-4 TG mice could be the result of the following possibilities: 1) The B cells may be arrested at a stage of development that is easily tolerizable, i.e., sIgM⁺IgD⁻. 2) The B cells may be unresponsive because of a previous encounter with autologous or environmental PC, which has resulted in a selective anergy or the programing of selective activation pathways. Other investigators have demonstrated that the B cells expressing endogenous IgM in M54 and Sp6 TG mice appear to be selected and can exhibit characteristics of sIg-activated cells (26–28). 3) There may be an activation defect common to all μκ TG mice, which results from transgene-induced alterations in B cell development. This latter possibility was addressed by stimulating spleen cells from Sp6 anti-TNP μκ TG mice (14) with soluble anti-μ. The data in Table VII show that both soluble anti-μ and anti-μ-conjugated Sepharose beads induced significant proliferation in both TG⁺ and TG⁻ B cell populations; thus, the defect in B cells from 207-4 mice is not simply the result of μκ transgene expression but may be related to either the site of the transgene integration or to the specificity of the B cells expressed in these mice. However, it is also possible that the anti-PC B cells in 207-4 TG mice arose from a different subset of B cells than the anti-TNP B cells in Sp6 TG mice, or that the anti-PC-specific B cells have been arrested at an early stage in their development.

Analysis of 207-4 B cells for Ly-1 and Mac-1 cell surface markers

Lamers et al. (15) have shown that Sp6 TG mice have low numbers of Ly-1 (CD5) B cells in their spleen and peritoneal cavity, whereas Herzenberg et al. (29) found that virtually all the B cells in M54 μ-only TG mice were of the B-1 phenotype (30). To further elucidate the nature of 207-4 TG⁺ B cells, the frequency of Ly-1 expression in the 207-4 anti-PC TG B cell population was examined by staining spleen and peritoneal cells from TG⁺ and TG⁻ littermates with FITC-conjugated polyvalent and

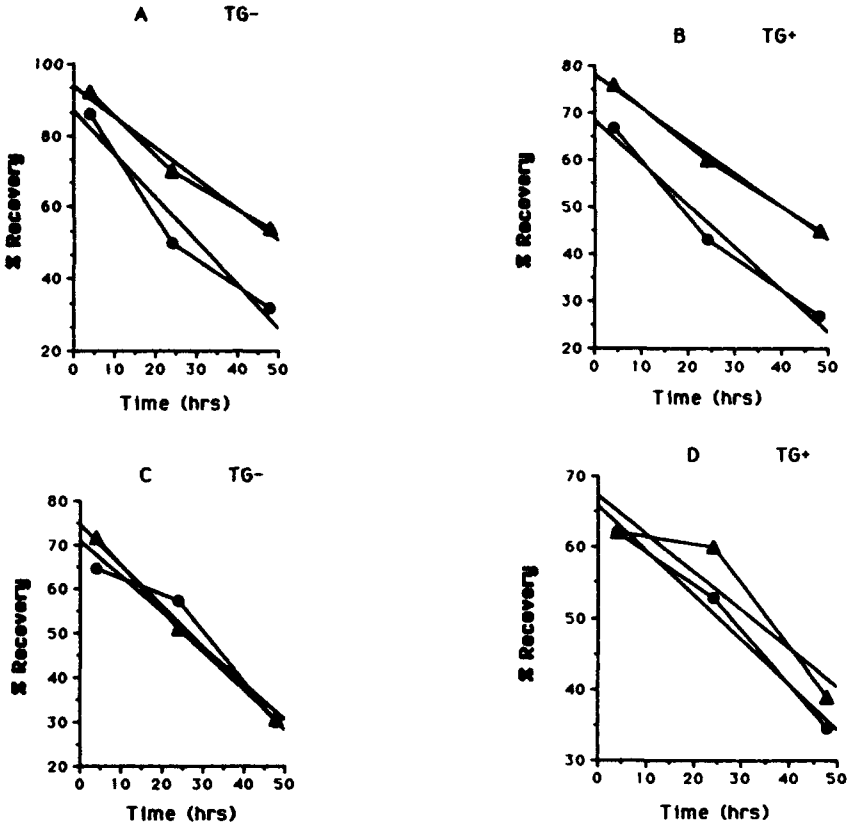

FIGURE 3. TG^+ and TG^- B cells die at the same rate in the presence of anti-μ Abs. In two separate experiments, purified TG^- B cells (*A* and *C*) and purified TG^+ B cells (*B* and *D*) were cultured in T25 flasks for 48 h at 10^6/ml in the presence (●) or absence (▲) of goat anti-μ Abs (100 μg/ml). Samples were taken at the times indicated and the viable cells were counted (% Recovery). Linear regression analysis was performed on the data from the two experiments, and is represented by the straight line over each plot. R^2 values range from 0.85 to 0.99 and the slope of the anti-μ-treated cells in each panel is slightly greater than that of the media control. Samples were also stained with FITC-anti-μ and PE-anti-B220. In all cultures, the percentage of B cells that stained with anti-B220 remained constant over the 48 h incubation period (data not shown).

Table VII. *Anti-μ-induced activation of spleen cells from Sp6 anti-TNP μκ transgenic mice*[a]

Stimulating Agent	TG^+	TG^-
	(cpm per culture × 10^{-3} (± SEM))	
Media	2.1 (±0.4)	4.5 (±0.2)
Anti-μ	47.3 (±4.4)	73.0 (±8.2)
Anti-μ-Sepharose	82.1 (±1.5)	130.0 (±8.9)
LPS	58.2 (±2.2)	95.8 (±3.1)
Con A	241.0 (±4.5)	255.0 (±5.6)

[a] Data represent the arithmetic mean (rounded to two or three significant figures) of the responses (geometric mean of triplicate cultures) of spleen cells from two TG^+ and two TG^- mice cultured individually.

monoclonal anti-IgM and biotin-conjugated anti-Ly-1 or anti-Mac-1 Abs. Because of the mouse-to-mouse variability in the cells from TG^+ mice, Table VIII includes the results obtained from four individual TG^+ mice. Less than 3% $Ly\text{-}1^+$ or $Mac\text{-}1^+$ B cells were observed in the spleens of either TG^+ or TG^- mice. Similar to the M54 transgenic mice analyzed by Stall et al. (31), the majority of peritoneal B cells from 207-4 anti-PC TG^+ mice expressed both the transgene encoded μ^a-allotype and the endogenous μ^b-allotype on their surface; however, unlike the M54 mice, less than 35% of the 207-4 peritoneal B cells express Ly-1, whereas 41 to 73% of these B cells express Mac-1. In the TG^- controls, Ly-1 and Mac-1 were observed on approximately 25% and 39% of the peritoneal B cells, respectively.

Discussion

The induction of proliferation of mouse B lymphocytes in vitro by anti-Ig Abs has been demonstrated in numerous studies to be a valuable tool for analyzing the ligand-receptor events associated with activation of B lymphocytes through their membrane IgM and IgD receptors. Activation of mouse B lymphocytes by anti-μ has been shown to be a function of a mature subset of $Lyb\text{-}5^+$ B cells (1). The data presented in this manuscript show that the M167 μκ TG mouse line, 207-4 Bri, has a defect in its B lymphocytes in that they cannot be stimulated to proliferate in vitro through their membrane IgM receptor by soluble anti-μ Abs. This unresponsiveness was demonstrated to be independent of the dose of anti-μ Ab used or the time of assay of the cultures, and was not overcome by costimulation with IL-4. Furthermore, depletion of T cells from the cultures by treatment with anti-Thy-1.2 plus C was unable to reverse this unresponsiveness (Table II), and coculture of TG^+ cells with TG^- cells did not induce any suppression in the TG^- cells in the same culture. The unresponsiveness was therefore not caused by active T cell suppression or any suppressive cytokine produced in the culture.

An unresponsiveness or diminished responsiveness of mouse B cells to soluble anti-Ig has been previously noted for 1) B cells of mice less than 8 wk of age (32), 2) CBA/N splenic B cells (32, 33), 3) B cell cultures stimulated with

Table VIII. *Analysis of B cell phenotype in anti-PC TG mice*[a]

Staining Agent	TG^+		TG^-	
	Spleen	PEC	Spleen	PEC
IgM^a:Ly-1	2.1	3.8		
	3.3	19.2		
	2.0	17.5		
	1.3	17.2		
IgM^b:Ly-1	<1.0	4.2	1.3	12.0
	1.1	23.0	1.7	8.7
	<1.0	16.1		
	1.1	10.7		
IgM^a:Mac-1	1.4	17.5		
	<1.0	27.1		
	<1.0	27.2		
	<1.0	27.2		
IgM^b:Mac-1	<1.0	17.4	<1.0	18.3
	<1.0	33.3	<1.0	13.4
	<1.0	29.1		
	<1.0	35.5		
Total IgM	27.6	35.7	24.5	53.5
	20.3	65.6	25.1	34.3
	13.2	58.9		
	11.2	48.6		

[a] Cells from the spleen and peritoneum of four TG^+ and two TG^- mice were stained and analyzed as described in *Materials and Methods*. Data represent the percent positive cells in the total cell population of each individual mouse.

rabbit anti-Ig Ab capable of suppressing the B cells through their FcR (3), and 4) B cells that have been preactivated in vivo (34–36). The unresponsiveness to anti-μ in the M167 $\mu\kappa$ TG mice, however, appears to be independent of the age of the animal (Fig. 1), and blocking of the FcR with the mAb 2.4G2 (20) did not overcome this unresponsiveness (Table II). Furthermore, $F(ab')_2$ preparations of goat anti-μ were not stimulatory for the B cells from these mice (Table III). These results demonstrated that unresponsiveness to anti-μ was not caused by FcR inhibition.

Although the B cells from 207-4 mice were unresponsive to soluble anti-μ, they could be stimulated by anti-μ, anti-Id, anti-allotype, or PC insolublized on Sepharose beads (Tables I and IV). A similar dichotomy between unresponsiveness to soluble anti-μ and responsiveness to insolublized anti-μ occurs with $Lyb\text{-}5^-$ B cells from *xid* mice (33), in neonatal B cells (37), and with normal B cells stimulated by monoclonal anti-μ (38). Although $Lyb\text{-}5^-$ B cells do not proliferate in response to soluble anti-μ treatment, they have been shown to increase in size (39) and to increase their membrane-associated Ia Ag (40). Similar to *xid* B cells, the 207-4 B cells also increase in size and increase their surface Ia (G. Azzolina and D. G. Sieckmann, unpublished observations); however, these $M167\text{-}Id^+$, PC-specific B cells are clonally deleted in *xid* mice (11) and therefore, they are not equivalent to $Lyb\text{-}5^-$ B cells. The spleen cells from 207-4 mice also lack both the Ly-1 and Mac-1 surface markers characteristic of $CD5^+$ B cells, although substantial numbers of $CD5^+$ B cells occur in their peritoneal cavity (Table VIII). B cells from 207-4 mice resemble B cells from neonatal mice (24, 25) and B cells that have recently emerged from the bone marrow of adult mice (12), in that they express high levels of sIgM and they lack sIgD, and thus, they could represent immature B cells. However, we have not been able to demonstrate the presence of the BP-3 alloantigen (12) on 207-4 splenic B cells (J. J. Kenny, unpublished observations), which suggests that they may not be equivalent to either of these types of B cells.

From the data presented in Table VI, it appears that a 1-h pulse of soluble anti-μ leads to death of many TG^+ B cells. However, when TG^+ and TG^- B cells were continuously stimulated with soluble anti-μ Abs (Fig. 3), there was no difference in their death rates. Thus from the present data, it seems that the failure of 207-4 TG^+ B cells to proliferate after anti-μ stimulation cannot be accounted for by induction of apoptosis in these cells. Nevertheless, it should be pointed out that the bulk culture conditions used for the cell recovery experiments under continuous or pulsed anti-μ exposure might not have provided the same culture conditions present in the 96-well cultures in which the 207-4 B cell stimulation defect was originally shown.

Indeed, interpretation of the results of these cell recovery experiments is additionally made difficult by the fact that cell numbers in these cultures are the result of simultaneous cell death and cell proliferation and, notably, by the fact that normal mature splenic B cells have been shown to undergo spontaneous apoptosis in unstimulated cultures (41). A more definitive study of the 207-4 B cell cultures is needed to examine for the presence of known biologic markers for apoptosis, such as chromatin condensation or DNA fragmentation. Along this line, a reduction in the size of many of the anti-μ-treated TG^+ B cells has been observed by flow cytometry (G. Nuñez, and J. J. Kenny, unpublished observations). In addition, introduction of the BCL-2 oncogene into the 207-4 mice has been shown to allow some of the TG^+ B cells to proliferate after stimulation with anti-μ (J. J. Kenny, unpublished observations).

Three alternate hypotheses could explain the restricted anti-μ stimulation defect observed in the B cells from 207-4 transgenic mice: 1) these B cells could be arrested at an immature stage of development, i.e., $sIgM^+IgD^-$; 2) this defect could be the consequence of a previous encounter with autologous or environmental PC, which has resulted in a restriction of the biochemical activation pathways that can be utilized on subsequent encounters with ligand; or 3) it could be an activation defect in these M167 $\mu\kappa$ TG mice, which results from transgene-induced alterations in B cell development. This defect is not common to all B cells from $\mu\kappa$ TG mice, because spleen cells from the $\mu\kappa$ anti-TNP Sp6 TG mice proliferate in response to both soluble anti-μ and anti-μ-conjugated to Sepharose beads

(Table VII). However, it is known that the TG^+ anti-TNP Sp6 TG B cells, which co-express endogenous sIgM, also express sIgD (14, 15). This suggests that the B cells in the TNP-TG mice may have fully matured, whereas, those in the M167 PC-specific TG mice may be immature. Of these alternate hypotheses, we favor the second. We have recently shown (9) that PC-specific cells are positively selected and expanded during their early development via a receptor-mediated process that appears to be Ag-driven. Inasmuch as PC is ubiquitous, i.e., it is present on the surface of both Gram-positive and Gram-negative bacteria, in the food and bedding of the mice, and also in autologous membranes, it appears likely that some type of thymus-independent event may be responsible for this selection. Hornbeck et al. (42) have recently demonstrated that the M167-Id^+ B cells from the 207-4 TG mice exhibit a fivefold higher level of phosphomyristin C than the B cells from their TG^- littermates. Phosphomyristin C is a principal phosphorylation substrate for protein kinase C, and it is induced in B cells after cross-linking of their sIgM receptors (43).

The fact that this sIgM receptor-inducible substrate is already elevated to levels seen in anti-μ-stimulated TG^- B cells suggests that the TG^+ B cells have previously undergone receptor-mediated signaling and may be anergic to soluble anti-μ cross-linking signals in the absence of appropriate cognate T cell help. Anti-μ-Sepharose may result in proliferation rather than cell death caused by signaling through alternate biochemical pathways as suggested by Brunswick et al. (44–46). We (6) and others (10) have shown that these M167-Id^+ B cells appear to respond normally in vivo and in vitro to thymus-dependent forms of PC; however, Pinkert et al. (47) found that only 1 in every 10^3 B cells from these 207-4 TG mice would respond in the splenic fragment assay. Chang et al. (48) have demonstrated that activated T cells are capable of preventing anti-μ-induced death in neonatal B cells; thus, the intervention of T cells via CD40 may be the normal physiologic event that prevents Ag-activated B cells from subsequent death.

The B cells from these M167 μκ anti-PC TG mice present a unique opportunity for elucidating the differences in the biochemical pathways that lead either to cell proliferation or cell death after signal transduction through the same Ig-receptor. High concentrations of soluble anti-μ have been shown to induce both intracellular and extracellular calcium transport and increased phosphatidylinositol (PI) turnover within minutes of sIgM receptor cross-linking (49). We have analyzed PI turnover in the B cells from these mice and demonstrated that this early activation event was not altered in these cells, i.e., there was no difference in PI turnover in TG^+ vs TG^- B cells after activation with anti-μ, LPS, or aluminum fluoride (21). Preliminary analysis of Ca^{+2}-flux in these cells also indicates that this activation step is unaltered, although initial unstimulated Ca^{2+} levels may be higher in the B cells from TG^+ mice (J. Mond, personal communication). These data may indicate that the initial biochemical pathways that ultimately lead to either the induction of proliferation or cell death after high dose anti-μ cross-linking of sIgM receptors are shared. Elucidating where these pathways diverge and distinguishing what determines which pathway will be utilized should lead to new insights on B cell development and regulation.

Acknowledgments

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References

1. Sieckmann, D. G. 1980. The use of anti-immunoglobulin to induce a signal for cell division in B lymphocytes via their membrane IgM and IgD. *Immunol. Rev. 52:181.*
2. Sieckmann, D. G., R. Asofsky, D. E. Mosier, I. Zitron, and W. E. Paul. 1978. Activation of mouse lymphocytes by anti-immunoglobulin. I. Parameters of the proliferative response. *J. Exp. Med. 147: 814.*
3. Parker, D. C. 1980. Induction and suppression of polyclonal antibody responses by anti-Ig reagents and antigen-nonspecific factors. *Immunol. Rev. 52:115.*
4. Storb, U. 1987. Transgenic mice with immunoglobulin genes. *Annu. Rev. Immunol. 5:151.*
5. Storb, U., C. Pinkert, B. Arp, P. Engler, K. Gollahon, J. Manz, W. Brady, and R. Brinster. 1986. Transgenic mice with μ and κ genes encoding anti-phosphorylcholine antibodies. *J. Exp. Med. 164: 627.*
6. Kenny, J. J., F. Finkleman, F. Macchiarini, W. C. Kopp, U. Storb, and D. L. Longo. 1989. Alteration of the B cell surface phenotype, immune response to phosphocholine and the B cell repertoire in M167 μ plus κ transgenic mice. *J. Immunol. 138:1363.*
7. Goodnow, C. C., J. Crosbie, S. Adelstein, T. B. Lavioe, S. J. Smith-Gill, R. A. Brink, H. Pritchard-Briscoe, J. S. Wotherspoon, R. H. Loblay, K. Raphael, R. J. Trent, and A. Basten. 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature 334:676.*
8. Nemazee, D. A., and K. Burki. 1989. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature 337:562.*
9. Kenny, J. J., C. O'Connell, D. G. Sieckmann, R. T. Fischer, and D. L. Longo. 1991. Selection of antigen-specific, idiotype positive B cells in transgenic mice expressing a rearranged M167-μ heavy chain gene. *J. Exp. Med. 174:1189.*
10. O'Brien, R. L., P. Marrack, U. Storb, and J. W. Kappler. 1988. B cells expressing Ig transgenes respond to a T-dependent antigen only in the presence of Ia-compatible T cells. *J. Immunol 141:3335.*
11. Kenny, J. J., A. M. Stall, D. G. Sieckmann, M. C. Lamers, F. Finkleman, L. Finch, and D. L. Longo. 1991. Receptor-mediated elimination of phosphocholine-specific B cells in X-linked immune-deficient mice. *J. Immunol. 146:2568.*
12. McNagy, K. M., P.-A. Cazenave, and M. D. Cooper. 1988. BP-3 alloantigen: A cell surface glycoprotein that marks early B lineage cells and mature myeloid lineage cells in mice. *J. Immunol. 141: 2551.*
13. Sidman, C. L., and E. R. Unanue. 1975. Receptor-mediated inactivation of early B lymphocytes. *Nature 257:149.*

14. Rusconi, S., and G. Köhler. 1985. Transmission and expression of a specific pair of rearranged immunoglobulin μ and κ genes in a transgenic mouse line. *Nature 314:330.*
15. Lamers, M. C., M. Vakil, J. F. Kearney, J. Langhorne, C. J. Paige, M. H. Julius, H. Mossmann, R. Carsetti, and G. Köhler. 1989. Immune status of a μ,κ transgenic mouse line: deficient response to bacterially related antigens. *Eur. J. Immunol. 19:459.*
16. Sieckmann, D. G., A. M. Stall, and B. Subbarao. 1991. A mouse monoclonal antibody specific for an allotypic determinant of the Igh^a allele of murine IgM: genetic and functional analysis of Igh-6a epitopes using anti-IgM monoclonal antibodies. *Hybridoma 10:121.*
17. Stall, A. M., and M. R. Loken. 1984. Allotypic specificities of murine IgD and IgM recognized by monoclonal antibodies. *J. Immunol. 132:787.*
18. Subbarao, B., and D. E. Mosier. 1983. Induction of B lymphocyte proliferation by monoclonal anti-Lyb-2 antibody. *J. Immunol. 130: 2033.*
19. Scribner, D. J., H. L. Weiner, and J. W. Morehead. 1978. Anti-immunoglobulin stimulation of murine lymphocytes. V. Age-related decline in Fc receptor-mediated immunoregulation. *J. Immunol. 121: 377.*
20. Unkeless, J. C. 1979. Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc-receptors. *J. Exp. Med. 150:580.*
21. Kenny, J. J., D. G. Sieckmann, C. Freter, R. Hodes, K. Hathcock, and D. L. Longo. 1992. Modulation of signal transduction in phosphocholine-specific B cells from $\mu\kappa$ transgenic mice. *Curr. Top. Microbiol. Immunol. 182:95.*
22. Howard, M., J. Farrar, M. Hilfiker, B. Johnson, K. Takatsu, T. Hamaoka, and W. E. Paul. 1982. Identification of a T cell-derived B cell growth factor distinct from interleukin 2. *J. Exp. Med. 155: 914.*
23. Rabin, E., J. Ohara, and W. E. Paul. 1985. B cell stimulatory factor (BSF-1) activates resting B cells. *Proc. Natl. Acad. Sci. USA 82: 2435.*
24. Vitetta, E. S., U. Melchers, M. McWilliams, M. E. Lamm, J. M. Phillips-Quagliata, and J. W. Uhr. 1975. Cell surface immunoglobulin. XI. The appearance of an IgD-like molecule on murine lymphoid cells during ontogeny. *J. Exp. Med. 141:206.*
25. Scher, I., A. K. Berning, S. Kessler, and F. D. Finkelman. 1980. Development of B lymphocytes in the mouse; studies of the frequency and distribution of surface IgM and IgD in normal and immune-defective CBA/N F1 mice. *J. Immunol. 125:1686.*
26. Forni, L. 1990. Extensive splenic B cell activation in IgM-transgenic mice. *Eur. J. Immunol. 20:983.*
27. Grandien, A., A. Coutinho, and J. Anderson. 1990. Selective peripheral expansion and activation of B cells expressing endogenous immunoglobulin in mu-transgenic mice. *Eur. J. Immunol. 20:991.*
28. Rabin, E. C. Ying-zi, T. Imanishi-Kari, and H. H. Wortis. 1992. Production of 17.2.25 μ transgenic and endogenous immunoglobulin in X-linked immune deficient mice. *Eur. J. Immunol. 22:2237.*
29. Herzenberg, L. A., A. M. Stall, J. Braun, D. Weaver, D. Baltimore, and R. Grosschedl. 1987. Depletion of the predominant B-cell population in immunoglobulin μ heavy-chain transgenic mice. *Nature 329:71.*
30. Herzenberg, L. A., A. B. Kantor, and L. A. Herzenberg. 1992. Layered evolution in the immune system: a model for the ontogeny and development of multiple lymphocyte lineages. *Ann. N. Y. Acad. Sci. 651:1.*
31. Stall, A. M., F. G. Kroese, F. T. Gadus, D. G. Sieckmann, and L. A. Herzenberg. 1988. Rearrangement and expression of endogenous immunoglobulin genes occurs in many murine B cells expressing transgenic membrane IgM. *Proc. Natl. Acad. Sci. USA 85:3546.*
32. Sieckmann, D. G., I. Scher, R. Asofsky, D. E. Mosier, and W. E. Paul. 1978. Activation of mouse lymphocytes by anti-immunoglobulin. II. A thymus-independent response by a mature subset of B lymphocytes. *J. Exp. Med. 148:1628.*
33. Mond, J. J., M. Schaefer, J. Smith, and F. D. Finkelman. 1983. Lyb-5$^-$ B cells of CBA/N mice can be induced to synthesize DNA by culture with insolubilized but not soluble anti-Ig. *J. Immunol. 131:2107.*
34. Cambier, J. C., C. H. Heusser, and M. H. Julius. 1986. Abortive activation of B lymphocytes by monoclonal anti-immunoglobulin antibodies. *J. Immunol. 136:3140.*
35. Cohen, D. P., and T. L. Rothstein. 1991. Elevated levels of protein kinase C activity and α-isoenzyme expression in murine peritoneal B cells. *J. Immunol. 146:2921.*
36. Ying-zi, C., E. Rabin, and H. H. Wortis. 1991. Treament of CD$^-$ B cells with anti-Ig, but not LPS, induces surface CD5: two B-cell activation pathways. *Int. Immunol. 3:467.*
37. Lindsberg, M.-L., M. Brunswick, H. Yamada, A. Lees, J. Inman, C. H. June, and J. J. Mond. 1991. Biochemical analysis of the immune B cell defect in xid mice. *J. Immunol. 147:3774.*
38. Udhayakumar, V., L. Kumar, and B. Subbarao. 1991. The influence of avidity on signaling murine B lymphocytes with monoclonal anti-IgM antibodies: effects on B cell proliferation versus growth inhibition (tolerance) of an immature B cell lymphoma. *J. Immunol. 146: 4120.*
39. DeFranco, A. L., J. T. Kung, and W. E. Paul. 1982. Regulation of growth and proliferation in B cell subpopulations. *Immunol. Rev. 64:161.*
40. Mond, J. J., E. Sehgal, and F. D. Finkelman. 1981. Increased expression of sIa on B lymphocytes after crosslinking of their surface immunoglobulin. In *Lymphocytes in the Immune Response.* Vol. II. N. Klinman, D Mosier, I. Scher, and E. Vittetta, eds. Elsevier/North Holland, New York. p. 177.
41. Illera, V. A., C. E. Perandones, L. L. Stunz, D. A. Mower, Jr., and R. F. Ashman. 1993. Apoptosis in splenic B lymphocytes. *J. Immunol. 151:2965.*
42. Hornbeck, P. V., S. P. Donald, and J. J. Kenny. 1992. Negative signalling through the B-cell antigen receptor. In *Progress in Immunology Vol. VIII, Proceedings of the Eighth International Congress of Immunology,* Budapest. J. Gergely, ed. Springer-Verlag, New York. p. 358.
43. Hornbeck, P., and W. E. Paul. 1986. Anti-immunoglobulin and phorbol ester induce phosphorylation of proteins associated with the plasma membrane and cytoskeleton in murine B lymphocytes. *J. Biol. Chem 261:14817.*
44. Brunswick, M., C. H. June, F. D. Finkelman, and J. J. Mond. 1989. Different patterns of inositol polyphosphate production are seen in B lymphocytes after cross-linking of sIg by anti-Ig antibody or by multivalent anti-Ig antibody dextran conjugate. *J. Immunol. 143:1414.*
45. Brunswick, M. E., E. Bonvini, M. Francis, C. C. Felder, T. Hoffman, and J. J. Mond. 1990. Absence of demonstrable phospholipid turnover in B cells stimulated by low mitogenic concentrations of anti-immunoglobulin. *Eur. J. Immunol.20:855.*
46. Brunswick, M., A. Burkhardt, F. Finkelman, J. Bolen, and J. J. Mond. 1992. Comparison of tyrosine kinase activation by mitogenic and nonmitogenic anti-IgD antibodies. *J. Immunol. 149:2249.*
47. Pinkert, C. A., J. Manz, P.-J. Linton, N. R. Klinman, and U. Storb. 1989. Elevated PC responsive B cells and anti-PC antibody production in transgenic mice harboring anti-PC immunoglobulin genes. *Vet. Immunol. Immunopathol. 23:321.*
48. Chang, T.-L., G. Capparo, R. E. Kleinman, and A. K. Abbas. 1991. Anergy in immature B lymphocytes. Differential responses to receptor-mediated stimulation and T helper cells. *J. Immunol. 147:750.*
49. Cambier, J. C., L. B. Justement, M. K. Newell, Z. Z. Chen, L. K. Harris, M. Sandoval, M. J. Klemsz, and J. T. Ramsom. 1987. Transmembrane signals and intracellular "second messengers" in the regulation of quiescent B-lymphocyte activation. *Immunol. Rev. 95:37.*